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There was an error published in *Development* **135**, 3197-3207 in the ePress and the online versions.

On p. 3204, the left- and right-hand columns of text were transposed.

The online issue has now been corrected. The print copy is correct.

We apologise to authors and readers for this mistake.

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A dual requirement for Iroquois genes during *Xenopus* kidney development

Pilar Alarcón*, Elisa Rodríguez-Seguel*, Ana Fernández-González, Ruth Rubio and José Luis Gómez-Skarmeta†

The Iroquois (Irx) genes encode evolutionary conserved homeoproteins. We report that *Xenopus* genes *Irx1* and *Irx3* are expressed and required during different stages of *Xenopus* pronephros development. They are initially expressed during mid-neurulation in domains extending over most of the prospective pronephric territory. Expression onset takes place after kidney anlage specification, but before pronephric organogenesis occurs. Later, during nephron segmentation, expression becomes restricted to the intermediate tubule region of the proximal-distal axis. Loss- and gain-of-function analyses, performed with specific morpholinos and inducible wild-type and dominant-negative constructs, reveal a dual requirement for *Irx1* and *Irx3* during pronephros development. During neurula stages, these genes maintain the specification of the pronephric territory and define its size. This seems to occur, at least in part, through positive regulation of Bmp signalling. Subsequently, Irx genes are required for proper formation of the intermediate tubule. Finally, we find that retinoic acid signalling activates both *Irx1* and *Irx3* genes in the pronephros.

KEY WORDS: Iroquois, Kidney, *Xenopus*, Regulation, Patterning

INTRODUCTION

Studies performed in different vertebrates indicate that most of the genes necessary for pronephros formation in *Xenopus* are also crucial for the formation of more complex mammalian metanephros or adult kidneys (Carroll et al., 1999; Dressler, 2006; Ryffel, 2003). Moreover, these similarities at the molecular level correlate with physiological homologies. Thus, the tubules of all nephrons have similar subdivisions along the anteroposterior axis with analogous distribution of transporters of small molecules and ions along this axis (Reggiani et al., 2007; Wingert et al., 2007; Zhou and Vize, 2004). This fact, and the accessibility of *Xenopus* to genetic manipulation, makes this animal an excellent model system with which to study kidney development. In *Xenopus*, the specification of the pronephric anlage occurs in the late gastrula/early neurula (stage 12). However, the first sign of pronephric morphogenesis, the thickening of the lateral mesoderm, is detected 10 hours later, in the late neurula (stage 20/21). At the tailbud stage (stage 25-30), differentiation of the three basic segments occurs: the corpuscle, the tubules and the duct. This is followed by the final maturation of the organ, which is associated with the physiological specialization of the pronephric tubules along the proximal-distal axis, as observed by the differential activation of several genes encoding different transport proteins (Carroll et al., 1999; Reggiani et al., 2007; Ryffel, 2003; Zhou and Vize, 2004).

The Iroquois (Irx) genes encode homeoproteins conserved during evolution with multiple functions during animal development (Gómez-Skarmeta and Modolell, 2002). Their role during patterning of the vertebrate nervous system has been studied in detail (Gómez-Skarmeta and Modolell, 2002). By contrast, their participation in the development of other organs is less well understood. Recently, it has been reported that *Xenopus Irx1*, *Irx2* and *Irx3* are expressed from the tailbud stage in the intermediate tubule segment of the pronephros, immediately prior to regionalization of the proximal-distal axis (Reggiani et al., 2007). This study further showed that *Irx3*, but not *Irx1* or *Irx2*, is required for development of this region (Reggiani et al., 2007).

We report that *Xenopus Irx1*, *Irx2* and *Irx3* are also expressed in the pronephric territory during earlier mid-neurula stages. Morpholino loss-of-function analyses, together with misexpression of inducible forms of wild-type and dominant-negative Irx proteins, reveal a two-step requirement for *Irx1* and *Irx3* during kidney development. Initially, *Irx1* and *Irx3* maintain the identity of the pronephric territory and define its size. This seems to occur, at least in part, through positive regulation of Bmp signalling. Later, both Irx genes are required for the formation of the intermediate tubule segment of the pronephros, as reported only for *Irx3* (Reggiani et al., 2007). In addition, we show that both genes are regulated by retinoic acid, which is known to be necessary to activate early kidney genes and to participate in the segmentation of the pronephros in the proximal-distal axis (Cartry et al., 2006; Wingert et al., 2007).

MATERIALS AND METHODS

Plasmid constructions

MT-Irx constructs

Constructs were made using Irx alleles from sequences AJ001834, AJ001835, AF027175, AF338157 and AF338158. *MT-Irx1*, *MT-Irx2* and *MT-Irx3* have been described previously (de la Calle-Mustienes et al., 2002). To generate *MT-Irx4* and *MT-Irx5*, a fragment from the 5′ region of each cDNA, including unique sites within the ORF (*Kpn*I in *Irx4* and *Cla*I in *Irx5*), were PCR amplified. These sites allowed us to fuse the PCR fragments to the rest of each cDNA. 5′ primers contained a *Xho*I (*Irx4*) or *Eco*RI (*Irx5*) site, allowing us to clone these PCRs fragments in frame within the pCS2-MT plasmid (Turner and Weintraub, 1994) at *Xho*I (*MT-Irx4*) or between *Eco*RI and *Xho*I (*MT-Irx5*). Primers used were: *Irx4*, 5′-cc**ctcgag**ATGTCATATCCTCAGTTTGGC-3′ and 5′-GCTCCCATCCA-TGGTACCATACC-3′; *Irx5*, 5′-gg**gaattc**aCATGTCCTATCCGCAGGGC-3′ and 5′-ATCCCCTGCATCTCCATC-3′. Bold nucleotides indicate restriction sites used for cloning procedures; capitals indicate sequences present in the cDNAs.

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Irx-MT constructs

To generate *Irx-MT* constructs, a fragment from the 3′ region of each cDNA, including unique sites within the ORF (*Sac*I in *Irx1*, *Pst*I in *Irx2*, *Sac*II in *Irx3*, *Kpn*I in *Irx4* and *Xba*I in *Irx5* cDNAs), were PCR amplified. For *Irx1*, *Irx2*, *Irx3* and *Irx5*, the fusion to the rest of each cDNA in pCS2-MT was carried out as follows: *Eco*RI/*Sac*I, *Pst*I, *Sac*II or *Xba*I fragments that contain the 5′ cDNA regions of *Irx1*, *Irx2*, *Irx3* or *Irx5*, respectively, were cloned into pBluescript. These fragments were excised with *Hin*dIII, *Sac*I, *Pst*I, *Sac*II or *Xba*I and ligated with the PCR fragment in pCS2-MT between the *Hin*dIII and *Cla*I (*Irx1*) or *Bam*HI (*Irx2*, *Irx3* and *Irx5*) sites. The full Irx ORF in frame with the Myc tag was then transferred into the *Eco*RI site of pCS2+. For Irx4, a *Cla*I/*Kpn*I fragment containing the 5′ cDNA was ligated with the corresponding 3′ PCR fragment, expanding the 3′ cDNA into the *Cla*I site of pCS2-MT. Primers used were: for *Irx1*, 5′-GCAACAA-GCCCAGATGG-3′ and 5′-cca**atcgat**GGCAGAGGGAAGTGCTG-3′; for *Irx2*, 5′-GCCGACCATCTTTGCG-3′ and 5′-gg**gggatcc**TGGGTATGG-TTGTACTCC-3′; for *Irx3*, 5′-CACAGCCCCATGTTCTGG-3′ and 5′-g**gggatcc**GGATGAGGATAAAGCGGA-3′; for *Irx4*, 5′-CCATGGTACCT-ACCCTCG-3′ and 5′-cca**atcgat**AGCAAGATGTTCTGTTCCT-3′; for *Irx5*, 5′-CTTCTCCATCTAGATCTCC-3′ and 5′-gg**gggatcc**AATGCTAG-ACATACCTTTCTTC-3′. Bold nucleotides indicate restriction sites used for cloning procedures; capitals indicate sequences present in the cDNAs.

MT-Irx-GR constructs

We first generated *Irx-MT-GR* derivatives by cloning the GR domain within the *Xho*I and *Xba*I fragment located 3′ of the *Irx-MT* in the pCS2 *Irx-MT* vectors. The hormone-inducible GR domain was obtained by PCR using the oligonucleotides 5′-ccc**ctcgag**ATCCCCTCTGAAAATCC-3′ and 5′-c**tctaga**CACTTTTGATGAAACAGAAG -3′ from a MyoD-GR plasmid kindly donated by H. Sive. To make the chimeric mRNAs of these constructs insensitive to the MOs, we introduced a MT 5′ by fusing these constructs with their corresponding *MT-Irx* as follows: for *Irx1*, a 5′ *Eco*RI-*Sac*I fragment from *MT-Irx1* was ligated to a 3′ *Sac*I-*Not*I fragment from *MT-Irx1-GR* in pCS2-MT; for *Irx2*, a 5′ *Eco*RI-*Apa*I fragment from *MT-Irx2* was ligated to a 3′ *Apa*I-*Not*I fragment from *MT-Irx2-GR* in pCS2-MT; for *Irx3*, a 5′ *Eco*RI-*Sac*II fragment from *MT-Irx3* was ligated to a 3′ *Sac*II-*Not*I fragment from *MT-Irx3-GR* in pCS2-MT.

In situ hybridization, X-Gal and antibody staining

Antisense RNA probes were prepared from cDNAs using digoxigenin or fluorescein labels (Roche). *Xenopus* specimens were prepared, hybridized and stained as described (Harland, 1991). X-Gal staining was performed accordingly to Coffman et al. (Coffman et al., 1993). Double fluorescent in situ hybridization was performed as previously described (Zhou and Vize, 2004). Antibody staining was performed as previously described (Gómez-Skarmeta et al., 2001). Monoclonal antibodies 3G8 and 4A6 were kindly provided by E. Jones. The monoclonal antibody 12/101, generated by J. P. Brockes, was obtained from the Developmental Studies Hybridoma Bank (NICHD and The University of Iowa, Department of Biological Science, Iowa City, IA 52242).

In vitro RNA synthesis, microinjection of mRNA and morpholinos, and grafts

DNAs were linearized and transcribed as described (Harland and Weintraub, 1985) with GTP cap analogue (New England Biolabs). SP6, T3 or T7 RNA polymerases were used. After DNAse treatment, RNA was extracted with phenol-chloroform, column purified and precipitated with ethanol. mRNAs for injection were resuspended in water. Synthetic mRNAs or MOs were injected into V2.2 blastomeres with 2-4 nl solutions. The following morpholinos were used in this study: MO*Irx1*, 5′-CATGTCTCTC-CGGCAGGGAAATCGC-3′; MO*Irx2*, 5′-AGGTAACCCTGAGGATA-GGACATGG-3′; MO*Irx3*, 5′-CTGTGGGAAGGACATGGTGCAGCCG-3′; MO*Irx3.2*, 5′-AGCTGTGGGAAGGACATGGTGCAGC-3′; MO*Irx4*, 5′-GTAGCCAAACTGAGGATATGACATT-3′; and MO*Irx5*, 5′-CAAGTAGCCCTGCGGATAGGACATG-3′. MO*Irx1* and MO*Irx5* are 100% homologous to the *Irx1* and *Irx5* alleles used in this study. The second *Irx5* and *Irx1* alleles contain 1 and 2 sequence mismatches, respectively, with their corresponding MOs. The other *Irx* MOs have 100% homology with all their corresponding *Irx* alleles. In the MO- or mRNA-injected embryos, in images taken at the same magnification, we used the histogram function of Photoshop to compare the size of the area expressing different markers in the injected versus the uninjected sides of the same embryo. Grafts were performed as previously described (Gómez-Skarmeta et al., 1999).

RESULTS

Expression patterns of Irx genes during *Xenopus* pronephros development

We examined the expression of the full complement of *Xenopus* Irx genes during pronephros development. *Irx1* and *Irx2* were largely co-expressed during pronephric development from late neurula onwards (Fig. 1A-H). *Irx3* was temporally and spatially expressed in similar, but not identical, territories (Fig. 1I-L). *Irx4* was only found in the pronephros at tadpole stage, *Irx5* was never detected in the kidney territory (see Fig. S1 in the supplementary material) and *Irx6* was not expressed at these stages (de la Calle-Mustienes et al., 2005). *Irx1*, *Irx2* and *Irx3* were initially expressed at mid neurula stages in the pronephric anlage (Fig. 1A,E,I). Double staining with the early pronephric markers *Lim1* and *Pax8* (Carroll and Vize, 1999; Heller and Brandli, 1999) confirmed that *Irx1* and *Irx3* were expressed in the pronephric field (insets in Fig. 1A,I; Fig. 1M,Q). Moreover, although the *Irx3* pronephric domain was broad and encompasses most, if not all, of the *Lim1* territory, *Irx1* was confined to the dorsal area of the *Lim1* field. At late neurula stage, *Irx1* was still confined to the dorsal pronephric domain (Fig. 1B,N). By contrast, *Irx3* became now restricted to the ventral pronephric territory (Fig. 1J,R). During tailbud stages, *Irx1* expression became localized in the intermediate tubule, as judged by double staining with *Pax8* (Fig. 1C, inset) or with the proximal tubule marker *Sglt1k* (Fig. 1O) (Reggiani et al., 2007; Zhou and Vize, 2004). At this stage, *Irx3* also became confined to the intermediate tubule, but its expression extended into the distal area of the proximal tubule and into the distal tubule, as determined by double staining with *Pax8* (Fig. 1K, inset) or with the intermediate tubule marker *Nkcc2* (Fig. 1S) (Reggiani et al., 2007; Zhou and Vize, 2004). The expression patterns of *Irx1* and *Irx3* were maintained at tadpole stages (Fig. 1D,L,P,T). In addition, late *Irx1* expression was also observed in migrating ventral mesoderm (Fig. 1D, blue arrowhead). We conclude that Irx genes have dynamic patterns of expression during pronephros development and that their expression in the pronephric territory starts much earlier than recently reported (Reggiani et al., 2007).

Loss of *Irx1* and *Irx3* function impairs pronephros development

To examine the requirement for Irx genes during *Xenopus* pronephros development, we interfered with the activity of each Irx mRNA by injecting specific translation-blocking morpholinos (MOs). As *Xenopus laevis* is pseudotetraploid, we identified all ESTs available in the database for each Irx gene and found one allele for *Irx4* and two alleles for *Irx1*, *Irx2*, *Irx3* and *Irx5*. We designed specific Irx MOs that block translation from both Irx alleles when present in the genome. The specificity of these MOs is shown in Fig. S2 in the supplementary material. Embryos injected with any of these Irx MOs showed different degrees of neural defects (E.R.-S., P.A. and J.L.G.-S., unpublished) indicating that they are effective in blocking the activity of their respective Irx genes. To reduce the MO effects on off-target tissues, in all experiments, we targeted the pronephric anlage by injecting the V2.2 blastomere of 8-16-cell stage embryos. We then evaluated the effect these injections on the early renal markers *Lim1* and *Pax8*. We considered that an embryo had an altered pronephros when the area expressing the corresponding marker on the injected

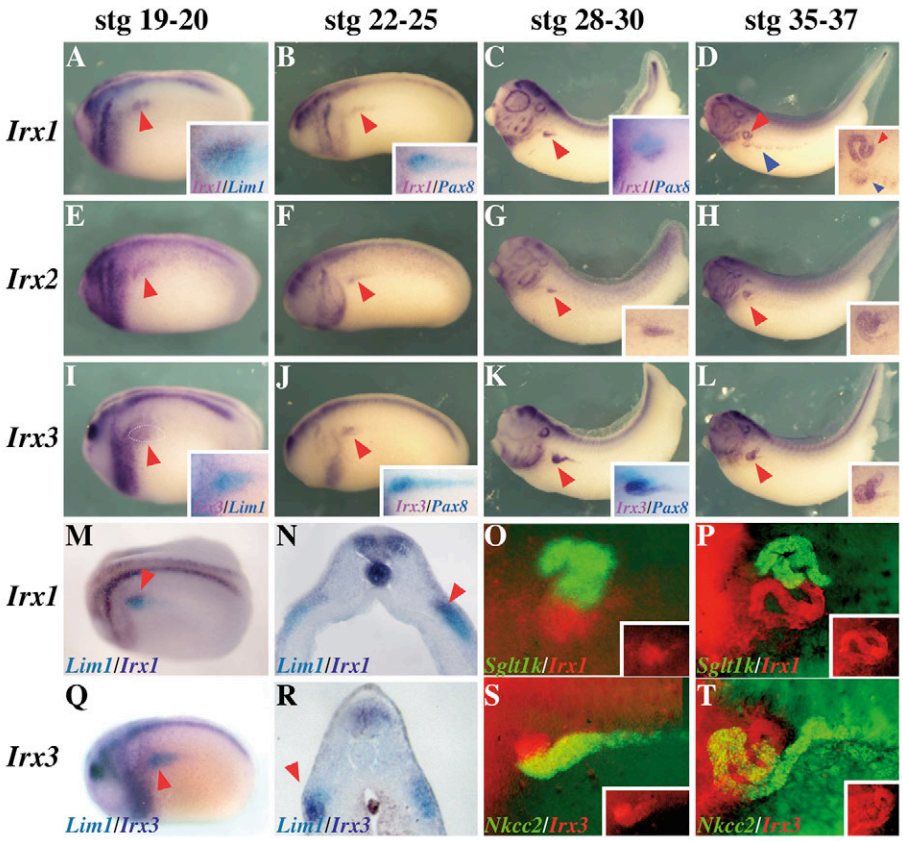

Fig. 1. Expression patterns of *Xenopus laevis* Irx genes during pronephros development. Embryos are shown in lateral views (except when indicated); red arrowheads indicate the kidney territory. (**A-D**) Expression pattern of *Irx1* at indicated developmental stages. At mid- (A) or late (B) neurula, *Irx1* is detected in the dorsal pronephric territory. Insets show pronephric territory of an embryo double-stained for *Lim1* or *Pax8* (blue) and *Irx1* (purple). *Irx1* expression is restricted dorsally. During tailbud (C) or tadpole (D) stages, *Irx1* expression shifts to a more ventral region that will form the intermediate tubule. Inset in C indicates an embryo double-stained for *Pax8* (blue) and *Irx1* (purple). Note the ventral position of *Irx1* in the future intermediate tubule. Inset in D indicates a higher magnification of the pronephric *Irx1* territory. Note the expression of *Irx1* in the migrating ventral mesoderm (blue arrowheads). (**E-H**) *Irx2* shows an expression pattern similar to that of *Irx1*, although it is not expressed in ventral migrating mesoderm. (**I-L**) Spatial distribution of *Irx3* mRNA. (I) At mid-neurula, *Irx3* mRNA is detected in a broad domain that contains most of the pronephric territory. Inset indicates pronephric territory of an embryo double-stained for *Lim1* (blue) and *Irx3* (purple). (J) At late neurula/early tailbud stages, *Irx3* becomes restricted to the ventral pronephric territory. Inset indicates *Irx3* ventral restriction in an embryo co-stained for *Pax8* (blue). (K,L) From tailbud stages, *Irx3* expression is detected in the intermediate tubule. Inset in K indicates embryo double-stained for *Pax8* (blue) and *Irx3* (purple). Inset in L indicates high magnification of the pronephric *Irx3* territory. (**M**,**N**) Lateral view (M) and transverse section (N) of late neurula embryos showing *Lim1* (blue) and *Irx1* (purple) expression. *Irx1* expression is restricted to the dorsal pronephric anlage. (**O**,**P**) Double staining for *Sglt1k* (green) and *Irx1* (red) in tailbud (O) or tadpole (P) embryos. Insets indicate single *Irx1* red channel. The *Irx1* expression domain is located just distal to that of *Sglt1k*. (**Q**,**R**) Lateral view (Q) and transverse section (R) of late neurula embryos showing *Lim1* (blue) and *Irx3* (purple) expression. There is initial broad expression of *Irx3* in most of the pronephric anlage (Q) and a later restriction to the ventral pronephros (R). (**S**,**T**) Double staining for *Nkcc2* (green) and *Irx3* (red) in tailbud (S) or tadpole (T) embryos. Insets show single *Irx3* red channel. The expression domains of both genes largely overlap, but the *Irx3* domain extends proximally into the proximal tubule, whereas *Nkcc2* extends distally into the distal tubule.

side varied by more than 20% relative to the uninjected control side. Differences greater that 20% between the two pronephros of a single embryo were very rarely observed in non-injected embryos or in embryos injected with a control MO (<2%, *n*=83). Injection of 8 ng of MOs against *Irx1* or *Irx3* (Fig. 2), but not *Irx2*, *Irx4*, *Irx5* or a control MO (Fig. S3 in the supplementary material; not shown), caused renal defects. Thus, at mid-late neurula stage, the territory expressing *Lim1* or *Pax8* was reduced in most *Irx1* or *Irx3* morphant embryos (Fig. 2A,B,I,J, and not shown). The average of pronephros size reduction was around 40-50%, and was observed in 57% (*n*=159) and 83% or (*n*=74) of the *MOIrx1*- and *MOIrx3*-injected embryos, respectively. As muscles are a source of signals that influence kidney development (Seufert et al., 1999), we determined whether, in the *Irx* morphant embryos, muscle development was altered. By staining with the muscle-specific antibody 12/101, we found that this was not the case (Fig. 2A,B,I,J). We also monitored the effect of injecting *MOIrx1* or *MOIrx3* on the expression of genes expressed at tadpole stages, during the maturation of the pronephros. All three genes examined, *Sglt1k*, *Nkcc2* and *Nbcc1* [which are expressed in proximal, intermediate and distal tubule, respectively (Reggiani et al., 2007; Zhou and Vize, 2004)], were downregulated in *MOIrx1* (38-47%, *n*=34-39) or *MOIrx3* (51-96%, *n*=24-41) morphants (Fig. 2C-H,K-P). The reduction of Irx function did not significant altered the rate of cell proliferation or cell death in the kidney territory (not shown).

We next examined the effect of knocking-down simultaneously both *Irx1* and *Irx3* (with a mix containing 3-4ng of each MO). Co-injection of Irx1 and Irx3 MOs at half doses caused phenotypes similar to individual MO injections at double concentration. The

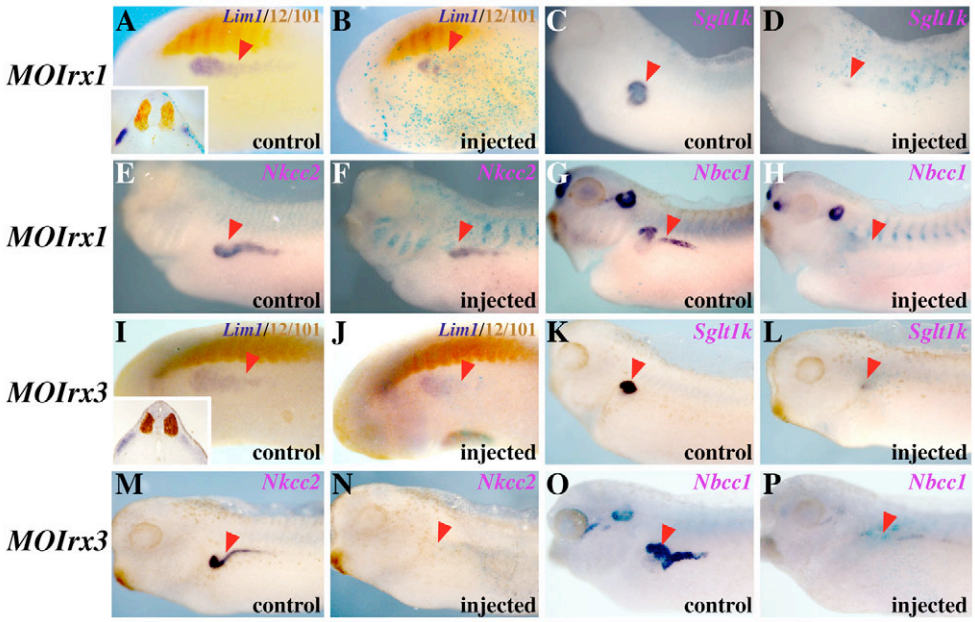

Fig. 2. *Irx1* and *Irx3* are necessary for kidney formation in *Xenopus*. Embryos are shown in lateral views; red arrowheads indicate the kidney territory. Embryos were injected in a single blastomere (V2.2) at the 8- to 16-cell stage and *lacZ* mRNA was used as linear tracer. Control and injected sides of the same embryo are shown, respectively, of the same specimen. The gene examined in each condition is indicated in the right upper corner of the panels in all figures. (**A-H**) Embryos injected with *MOIrx1* showed reduced *Lim1* expression at late neurula (A,B) and downregulation of *Sglt1k* (C,D), *Nkcc2* (E,F) and *Nbcc1* (G,H) expression at tadpole stages. Inset in (A) indicates a transverse section of the embryo shown in the major panel. (**I-P**) Similar results were found upon *MOIrx3* injection.

simultaneous impairment of *Irx1* and *Irx3* resulted in the loss of *Lim1* (58%, *n*=66) and *Pax8* (89%, *n*=57) expression only at late neurula stage, coinciding with the onset of expression of *Irx1* and *Irx3*, but it did not affect the expression of these genes at early neurula (Fig. 3A-F). In these injected embryos, the differentiated phronephric structures at tadpole stages, but not the somitic muscles, were severely reduced, as determined by triple staining with the antibodies 3G8 and 4A6, which label the tubules and the duct, respectively (Vize et al., 1995), and 12/101 (Fig. 3G-J). Interestingly, in these Irx-depleted embryos, the number of ventral muscle fibres was reduced (Fig. 3G,H, blue arrowheads). This suggests that the expression of *Irx1* in ventral migrating muscle cells may be required for proper development of these muscles. Sections through these injected embryos suggested that cells that lose their kidney fate are likely to end up as fibroblasts, as an increased number of cells with fibroblast shape are detected in the *Irx* depleted side (Fig. 3J).

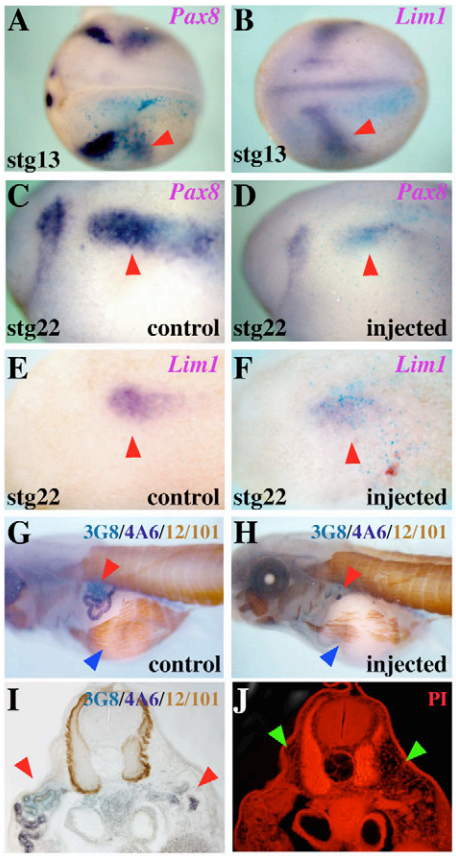

Fig. 3. Irx genes are not required for the initial activation of the early kidney genes. Embryos are shown in lateral views (except A and B, which are dorsal views); red arrowheads indicate the kidney territory. Embryos were injected in a single blastomere (V2.2) at the 8- to 16-cell stage. *Xenopus* embryos injected with a mix of *Irx1* and *Irx3* MOs and *lacZ* mRNA were assayed for the expression of *Pax8* and *Lim1* genes at early (A,B) or late (C-F) neurula stages. (**A**,**B**) Impairment of Irx gene function does not affect early expression of *Pax8* (A) or *Lim1* (B). (**C-F**) By contrast, depletion of *Irx* activity downregulates the expression of these genes at late neurula stage. (**G**,**H**) Tadpole embryos injected with *Irx1* and *Irx3* MOs and triple labelled for muscle (12/101, brown), pronephric tubules (3G8, blue) and duct (4A6, purple). The injected side (H) shows strong impairment of kidney tissue (red arrowheads) and reduced number of ventral muscle fibres (blue arrowheads) when compared with the control side (G). (**I**) Transverse section of the embryo shown in H. (**J**) The same section after treatment with propidium iodide. An increased number of fibroblast-like cells in the injected right side compared with the control left side (arrowheads).

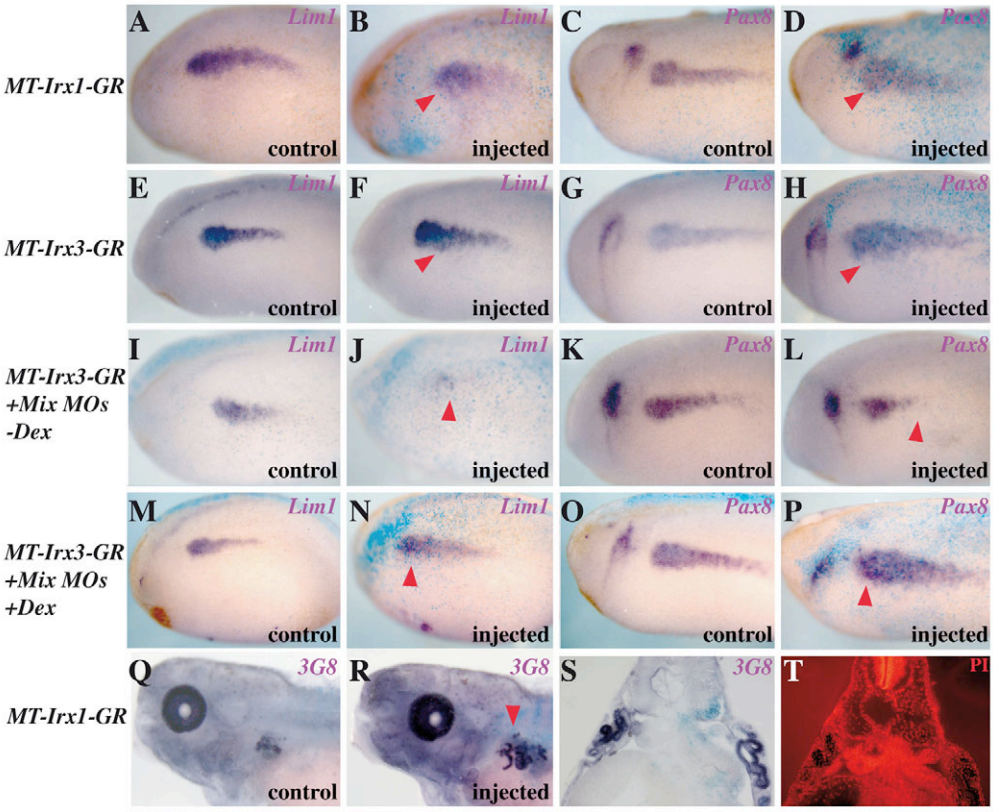

Fig. 4. Overexpression of Irx genes in *Xenopus* expands the pronephric territory. Embryos are shown in lateral views (except when indicated). Embryos were injected in a single blastomere (V2.2) at the 8- to 16-cell stage and *lacZ* mRNA was used as linear tracer. Neurula (A-P) or tadpole (Q-R) embryos injected with different mRNAs. Transverse sections of tadpole embryos are shown in S,T. (**A-H**) Overexpression of 300 pg of *MT-Irx1-GR* (A-D) or *MT-Irx3-GR* (E-H) mRNAs expands (arrowheads) ventrally the expression of *Lim1* (A,B,E,F) and *Pax8* (C,D,G,H) upon addition of dexamethasone (Dex) at stage 14, whereas no effect was observed in the absence of Dex (not shown). (**I-P**) Embryos co-injected with a mix of *Irx1* and *Irx3* MOs and *MT-Irx3-GR* mRNAs show strong downregulation of *Lim1* (I,J; arrowhead) and *Pax8* (K,L; arrowhead) in the absence of Dex. (M-P) This phenotype is rescued upon addition of hormone at stage 14. (**Q-T**) Tadpole embryos injected with *MT-Irx1-GR* mRNAs and Dex treated at stage 14 show enlarged kidneys (Q,R; arrowhead), as determined by staining with the 3G8 antibody. (S) Transverse section of a similarly injected embryo. (T) The same section treated with propidium iodide for nuclear staining. The control and the enlarged pronephros show the same cellular morphology.

The autonomous requirement of *Irx1* and *Irx3* for pronephros formation was further supported by a transplantation experiment. A graft of lateral plate from a late gastrula embryo co-injected with *Irx1MO*, *Irx3MO* and *GFP* mRNA was transplanted to the equivalent area of a wild-type host. In the transplanted embryo, the expression of *Lim1*, but not that of neural or muscle markers (*Sox2* and 12/101, respectively), was impaired (see Fig. S4 in the supplementary material) (66%, n=6). This was not observed after transplantation of a control graft from an embryo injected with only *GFP* mRNA (100%, n=7; not shown). Finally, we also monitored the expression of several additional kidney markers (*Osr2*, *Nhf1β*, *Wnt4* and *Wt1*) in double *Irx1* and *Irx3* morphant embryos. Expression of all genes was reduced (see Fig. S5 in the supplementary material). Together, our results indicate that *Irx1* and *Irx3* are activated following the specification of the kidney anlage and are autonomously required for the maintenance of this specification.

Gain of *Irx1* and *Irx3* function expand the pronephric field

Our results prompted us to test the effect of the misexpression of Irx genes. We first generated hormone-inducible forms of the *Irx1*, *Irx2* and *Irx3* proteins (MT-Irx-GR) that are insensitive to the MOs (see Materials and methods). These constructs allowed the induction of Irx function after gastrulation, thus eliminating possible earlier effects of Irx genes on mesoderm formation (Glavic et al., 2001). All three MT-Irx-GR proteins behaved similarly in overexpression studies (see below and not shown). Consistent with a requirement for Irx genes during pronephric development, overexpression of *MT-Irx1-GR* or *MT-Irx3-GR* mRNAs, upon addition of dexamethasone (Dex) at stage 14, triggered a ventral expansion of *Lim1* and *Pax8* (Fig. 4A-H; 60% of the embryos showing enlarged pronephros, n=132). In most embryos, the pronephros at the injected side was about 50% larger than the pronephros at the control non-injected side. This expansion enlarged the differentiated kidney tissue, as determined by staining with the tubules antibody (3G8) (Fig. 4Q-T). We next determined the ability of these MT-Irx-GR constructs to rescue the defects observed in Irx morphant embryos. Although interference with *Irx1* and *Irx3* function with a mix of MOs caused downregulation of *Lim1* and *Pax8* (Fig. 4I-L; Fig. 3C-F), co-injection of *Irx* MOs with *MT-Irx1-GR* or *MT-Irx3-GR* mRNAs rescued the expression of these genes, but only upon hormone addition at early neurula stage (Fig. 4M-P and not shown; 15% reduced and 55% enlarged pronephros, n=100).

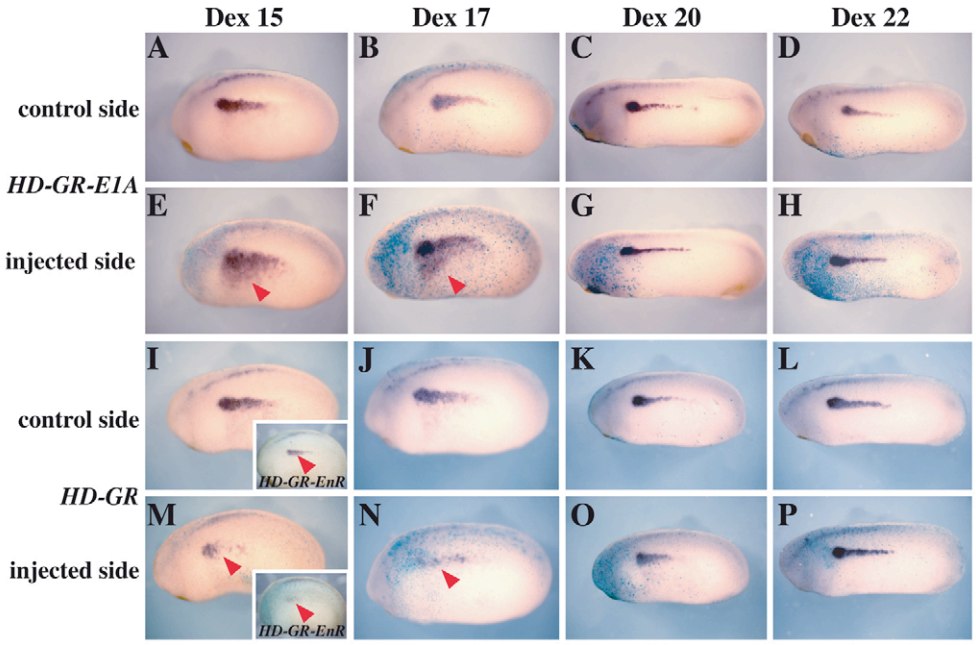

Fig. 5. Early Irx gene requirement for pronephros development occurs at neurula stages. Embryos are shown in lateral views. Embryos were injected in a single blastomere (V2.2) at the 8- to 16-cell stage and *lacZ* mRNA was used as linear tracer. Late neurula-early tailbud *Xenopus* embryos co-injected with 500 pg of *HD-E1A-GR* (A-H), *HD-GR* (I-P) or *HD-EnR-GR* (I,M, inset) mRNAs and assayed for expression of *Lim1*. (**A-H**) Embryos injected with a hormone-inducible activating form of Irx (HD-E1A-GR) show expanded *Lim1* only when Dex was added during mid neurula stages (arrowheads). (**I-P**) Embryos injected with a hormone-inducible dominant negative (HD-GR) form of Irx show reduced *Lim1* expression only when Dex was added during mid neurula stages (arrowheads). The same results were found with a hormone-inducible repressing form of Irx (HD-EnR-GR) (I and M, inset and not shown).

These results indicate that the pronephric expression of Irx genes is required to maintain the transcription of the key renal genes *Lim1* and *Pax8*, and to define the size of the pronephric anlage.

During neurula stage Irx proteins act as activators in the pronephric field

To cast light on the way Irx proteins function during these processes, we overexpressed different hormone-inducible constructs with the homeodomain (HD) of Irx1 alone or fused to either the E1A activator or the Engrailed (EnR) repression domains (HD-GR, HD-GR-E1A and HD-GR-EnR). It is known that, during neural development, Irx proteins act as transcriptional repressors, as overexpression of wild-type Irx proteins or HD-GR-EnR fusions cause similar phenotypes, whereas HD-GR and HD-GR-E1A behave as dominant-negative molecules (Gómez-Skarmeta et al., 2001). By contrast, during kidney development, overexpression of *HD-GR-E1A* mRNA (Fig. 5A-H) mimicked the ventral expansion of *Lim1* (64% of the embryos with enlarged pronephros, n=48) caused by wild-type Irx mRNAs (Fig. 4). Conversely, the overexpression of *HD-GR* (Fig. 5I-P) or *HD-GR-EnR* (inset in Fig. 5I, M, and not shown) mRNAs promoted downregulation of *Lim1* (88% of the embryos showing reduced pronephros, n=54). Therefore, during pronephros development, HD-GR and HD-GR-EnR proteins behave as dominant-negative molecules that interfere with Irx function. Similar results were found when *Pax8* expression was examined (not shown). In addition, by providing Dex at different stages of development, we also found that the requirement for Irx function during pronephros development occurred around mid neurula (stages 15-17). Overexpression of Irx proteins at later stages (20-22) had little effect on *Lim1* and *Pax8* (Fig. 5). These data suggest that Irx proteins act as transcriptional activators to maintain the kidney

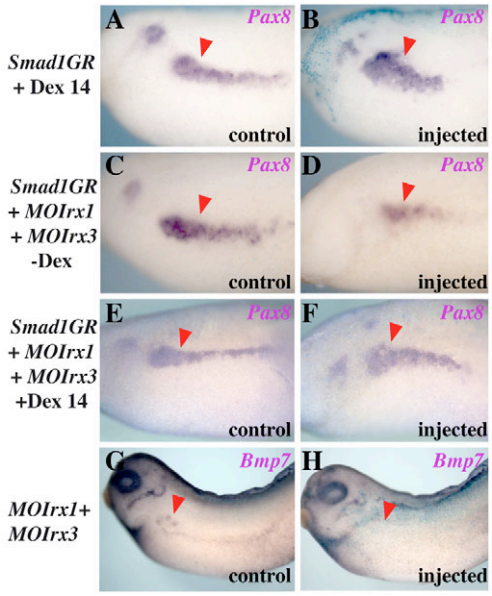

Fig. 6. Irx gene loss of function kidney defects are partially rescued by increased Smad1 activity in *Xenopus*. Embryos are shown in lateral views and red arrowheads indicate the kidney territory. Embryos were injected in a single blastomere (V2.2) at the 8- to 16-cell stage and *lacZ* mRNA was used as linear tracer. (**A**,**B**) Injection of 500 pg of *Smad1GR* mRNA, upon addition of dexamethasone (Dex) at stage 14, expanded *Pax8* expression. No effect was observed in the absence of hormone (not shown). (**C-F**) In embryos co-injected with 500 pg of *Smad1GR* mRNA and 4 ng of each *Irx1* and *Irx3* MOs *Pax8* expression was downregulated (C,D) or rescued (E,F) in the absence or presence of Dex, respectively. (**G**,**H**) Depletion of *Irx1* and *Irx3* impaired *Bmp7* expression.

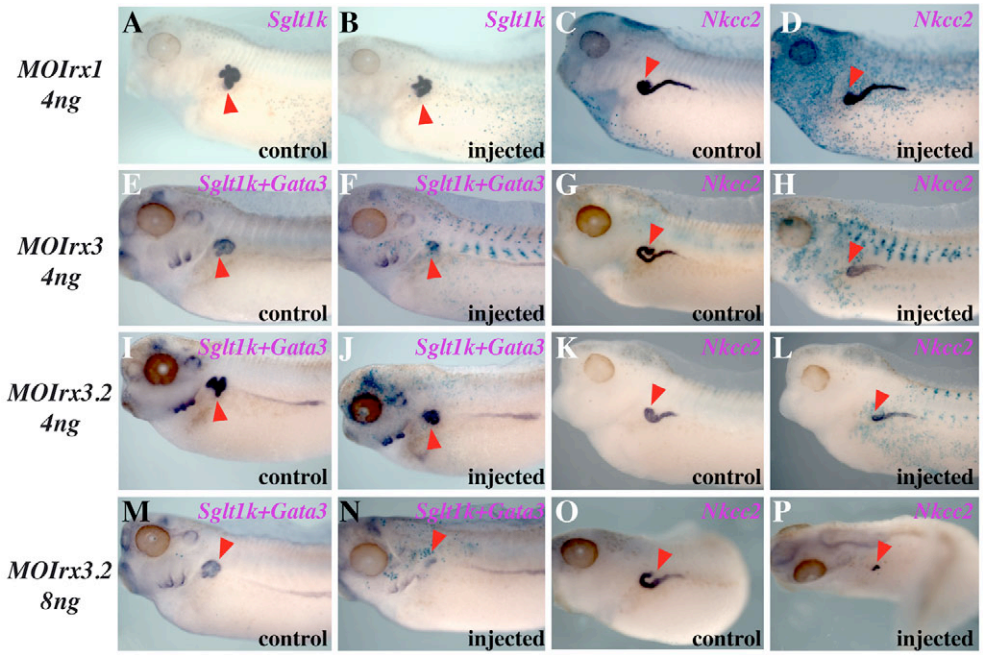

Fig. 7. Injection of different doses of *Irx1 or Irx3* MOs reveal an early and a late requirement of this gene during pronephric development in *Xenopus*. Embryos are shown in lateral views and red arrowheads indicate the kidney territory. Embryos were injected in a single blastomere (V2.2) at the 8- to 16-cell stage and *lacZ* mRNA was used as linear tracer. (**A-D**) Injection of low doses (4 ng) of *Irx1* MO had little effect on *Sglt1k* expression (A,B) but downregulated the proximal domain of *Nkcc2* (C,D). (**E-L**) Injection of low doses (4 ng) of two different *Irx3* MOs downregulated the distal expression of *Sglt1k* (E,F,I,J) and the proximal domain of *Nkcc2* (G,H,K,L). No effect was observed in the duct, as determined by *Gata3* expression (E,F,I,J). (**M-P**) Injection of high doses (8 ng) of *MOIrx3.2* strongly downregulated *Sglt1k* (M,N) and *Nkcc2* (O,P). Most injected embryos were malformed, as shown in O,P. Nevertheless, a few displayed normal morphology, like that shown in M,N.

anlage properly and to define the size of this territory, and confirm that they are initially required before pronephros morphogenesis takes place.

Rescue of Irx-dependent kidney defects by increasing Bmp signalling

The Bmp pathway is implicated at several steps during vertebrate kidney development (Cain et al., 2008). A recent report showed that blocking this pathway during *Xenopus* neurula stages impaired pronephros formation (Bracken et al., 2008). These results resemble those observed by reducing Irx function. As Irx genes modulate Bmp signalling during neural development (Gómez-Skarmeta et al., 2001), a similar situation may occur during kidney development. To evaluate this, we increased Bmp signalling at neurula stages, by overexpressing an inducible Smad1 construct (Smad1GR) in the absence of *Irx1* and *Irx3* activity. Induction of Smad1GR at stage 14 caused an expansion of kidney territory (51%, *n*=35), as determined by *Pax8* expression (Fig. 6A,B). In the absence of Dex, embryos co-injected with *Smad1GR* mRNA and *Irx1* and *Irx3* MOs showed downregulation of *Pax8* (Fig. 6C,D; 41%, *n*=51). This phenotype is partially rescued by Dex treatment at stage 14 (Fig. 6E,F; 21% with *Pax8* downregulated, *n*=48). Thus, part of Irx function seems to be to positively modulate the Bmp pathway. *Bmp7* is expressed and required for kidney development (Dudley et al., 1995; Luo et al., 1995; Wang et al., 1997). We examined whether its expression depended on Irx activity. Indeed, as for other kidney markers, in double *Irx1* and *Irx3* morphant embryos *Bmp7* expression was downregulated (Fig. 6G,H).

Irx1 and *Irx3* genes are required for proximal-distal patterning of the pronephros

Irx3, but not *Irx1*, has been shown to be required for formation of the intermediate tubule segment of the pronephros (Reggiani et al., 2007). We examined whether *Irx1* was also required in this late process. Injection of high doses of *Irx* MOs downregulated all proximal-distal genes, probably because of the early requirement of Irx genes for maintaining the kidney anlage, thus preventing examination of later Irx functions. To try to overcome this problem, we partially reduced Irx function by injecting *Irx1* and *Irx3* MOs at lower doses. We complement these experiments injecting a second *Irx3* MO (*MOIrx3.2*) that, in a previous report, was unable to reveal an early *Irx3* requirement (Reggiani et al., 2007). Blastomere injection of 4 ng of *Irx1* MO or either one of the two *Irx3* MOs caused little effect on *Lim1* and *Pax8* expression in late neurula or tailbud stages (not shown). By contrast, at tadpole stage, embryos injected with *Irx1* or with any of the *Irx3* MOs showed downregulation of the proximal domain of *Nkcc2* (40-50%, *n*=19-26) (Fig. 7C,D,G,H,K,L). This was also accompanied by a reduction in the distal expression of *Sglt1k* in the *MOIrx3*, but not in the *MOIrx1*, injected embryos (Fig. 7A,B,E,F,I,J). These results are consistent with the expression domains of *Irx1* and *Irx3*, and suggest that both genes are required for proximal-distal patterning, as was already reported for *Irx3* (Reggiani et al., 2007). We also tried to address why, in the previous report (Reggiani et al., 2007), an early requirement for *Irx3* was not detected. For that, we injected one of the *Irx3* MO they used (*MOIrx3.2*) at higher (8 ng) doses. At this concentration, most embryos injected with our *Irx3* MO were

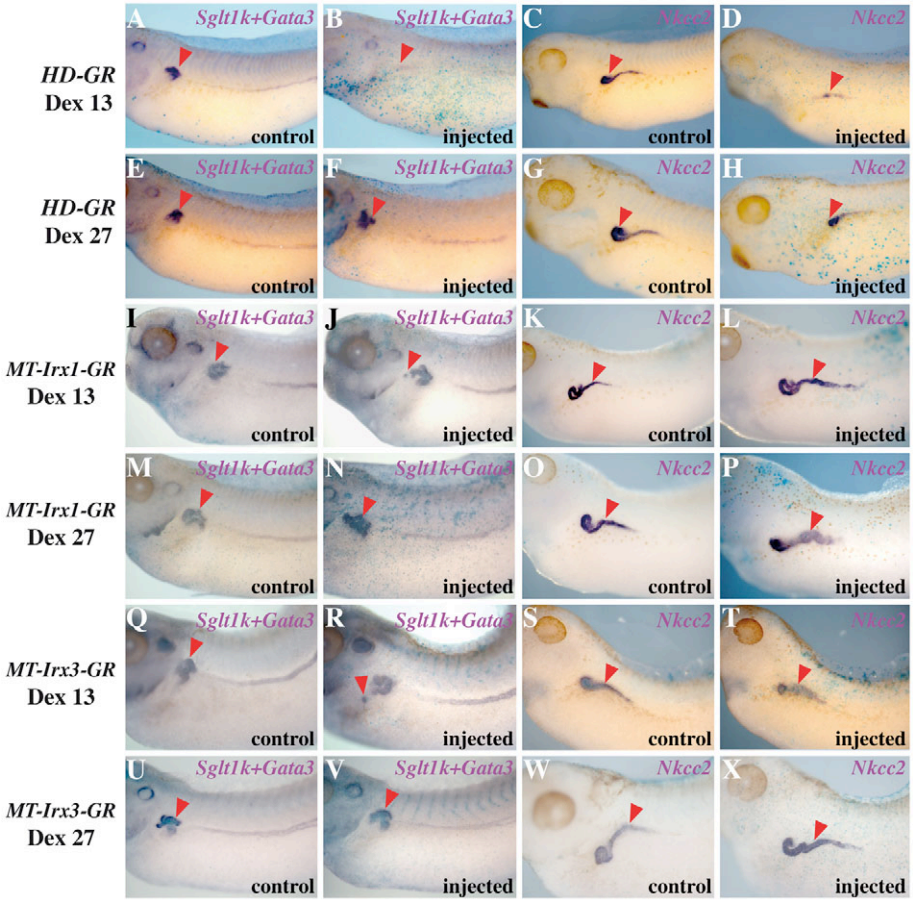

Fig. 8. Temporal requirement for Irx function during proximal-distal pronephric patterning. Embryos are shown in lateral views and red arrowheads indicate the kidney territory. Embryos were injected in a single blastomere (V2.2) at the 8- to 16-cell stage and *lacZ* mRNA was used as linear tracer. (A-H) *Xenopus* embryos injected with *HD-GR* mRNA. (**A-D**) Impairment of *Irx* activity during neurula stages downregulated *Sglt1k, Gata3* (A,B) and *Nkcc2* (C,D) expression. (**E-H**) Impairment of *Irx* activity during tailbud stages did not affect *Sglt1k* or *Gata3* (E,F) but reduced *Nkcc2* (G,H) expression. (**I-P**) Embryos injected with *MT-Irx1-GR* mRNA. (I-L) Increasing *Irx1* function during neurula caused ectopic, patched *Sglt1k* (I,J) and enlarged *Nkcc2* expression domains (K,L). No effect was observed on the duct marker *Gata3* (I,J). (M-P) Increasing *Irx1* function during tailbud did not affect *Sglt1k* or *Gata3* (M,N) but enlarged *Nkcc2* (O,P) expression. (**Q-X**) Similar results were found in embryos injected with *MT-Irx3-GR* mRNA.

healthy and showed the strong reduction of all segment markers shown in Fig. 2. By contrast, the majority of the embryos injected with 8 ng of *MOIrx3.2* were malformed. These malformed embryos also lost most markers (Fig. 7M-P). This might explain the discrepancy if those embryos with stronger phenotypes and malformations were not taken into account in the previous report (Reggiani et al., 2007).

To further examine the Irx requirement in pronephric proximal-distal patterning, we injected inducible wild-type or dominant-negative Irx constructs, activated them at different developmental stages and examined their effect on pronephric proximal-distal patterning. A dominant-negative Irx construct induced at stage 13 downregulated *Sglt1k*, *Nkcc2* and *Gata3* (84-90%, *n*=19-20) (Fig. 8A-D), markers of proximal tubule, intermediate tubule and duct, respectively (Reggiani et al., 2007; Wingert et al., 2007; Zhou and Vize, 2004). By contrast, the same construct induced at stage 27 downregulated *Nkcc2* (59%, *n*=17), whereas *Sglt1k* and *Gata3* were not affected (Fig. 8E-H). These results are consistent with an early and a late requirement for Irx function. A further confirmation of this dual function was obtained by examining these proximal-distal markers in embryos injected with Dex-inducible Irx1 or Irx3 proteins. Incubation of injected embryos with Dex from stage 13 caused ectopic patches of *Sglt1K* (38-47%, *n*=15-24) (Fig. 8I,J,Q,R) and an enlarged *Nkcc2* domain (52-60%, *n*=17-24) (Fig. 8K,L,S,T). No clear effect was found on *Gata3* expression (Fig. 8I,J,Q,R). Addition of hormone at stage 27, expanded the *Nkcc2* expression domain (50-60%, *n*=14-22) (Fig. 8O,P,W,X), but did not affect *Sglt1K* expression (Fig. 8M,N,U,V).

Retinoic acid regulates pronephric expression of *Irx1* and *Irx3*

Retinoic acid (RA) is a requisite for the activation of early kidney genes and for the late segmentation of the pronephros (Cartry et al., 2006; Wingert et al., 2007). Therefore, RA may regulate Irx genes during kidney development. To test this, we examined Irx expression in embryos injected with 100 pg of mRNAs encoding either a dominant-negative (RAR-DN) or a constitutively active (RAR-Vp16) RA receptor (Blumberg et al., 1997). Embryos with reduced or increased RA signalling showed down or upregulation, respectively, of the expression of Irx genes in the kidney (Fig. 9A-H). Thus, RA positively regulates Irx genes in the pronephros. We then determined when RA signalling is required for kidney Irx expression. To this end, we incubated *Xenopus* embryos at different developmental stages (12.5, 15 or 25) for 1 hour with a control solution (DMSO), with 4-diethylaminobenzaldehyde (DEAB 30 □M), the chemical inhibitor of the RA producing enzyme Raldh2 or with RA (10 □M). Reducing or increasing RA signalling, down or upregulated, respectively, the expression of both genes, but only when the drug treatments were done at late gastrula stage (12.5) (Fig. 9I-Y).

DISCUSSION

The development of the pronephros can be subdivided in three major steps: specification of the anlage, morphogenesis of the nephron and the generation of different proximal-distal territories. Here, we show that the homeodomain genes *Irx1* and *Irx3* play essential functions in two of these steps: maintenance of the specified anlage and segmentation of the nephron.

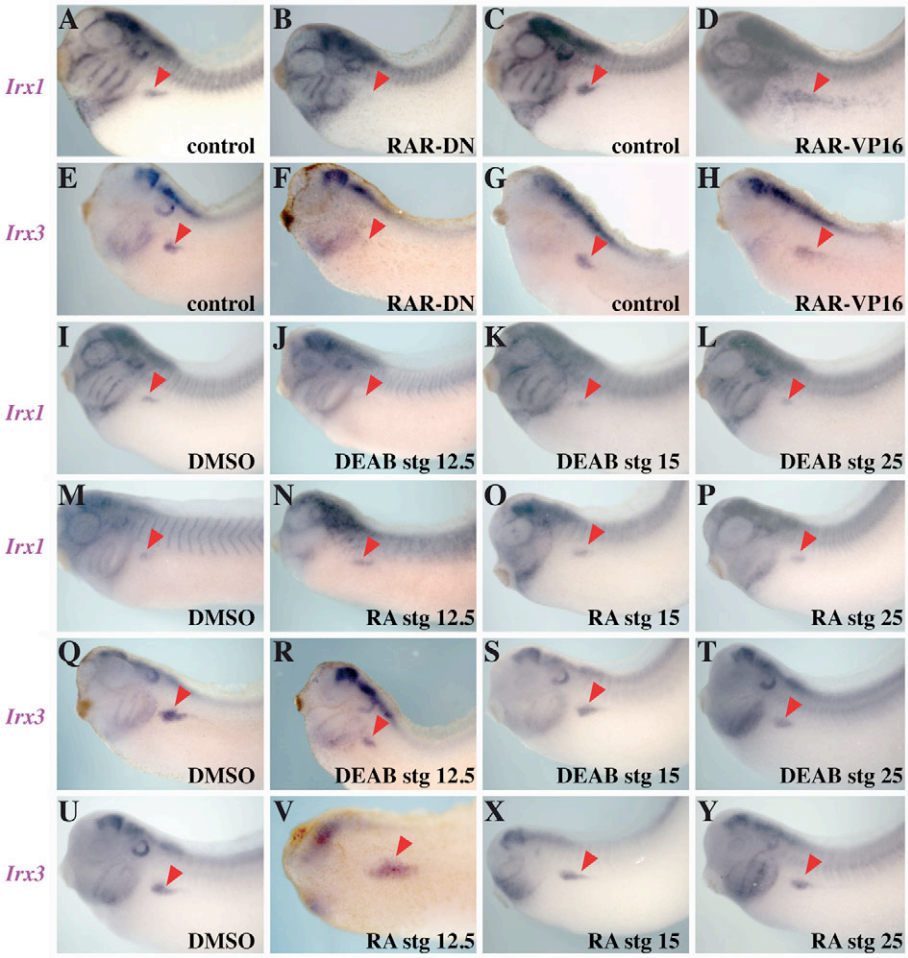

Fig. 9. *Irx1* and *Irx3* are positively regulated by retinoic acid signalling. Embryos are shown in lateral views and red arrowheads indicate the kidney territory. Embryos were injected in a single blastomere (V2.2) at the 8- to 16-cell stage and *lacZ* mRNA was used as linear tracer. All panels show *Xenopus* tadpole embryos. (**A**,**B**,**E**,**F**) Embryos injected with a dominant negative RA receptor mRNA (RAR-DN) showed impaired *Irx1* (A,B) and *Irx3* (E,F) expression. (**C**,**D**,**G**,**H**). Embryos injected with a constitutive RA receptor mRNA (RAR-VP16) showed a strong expansion of *Irx1* (C,D) and *Irx3* (G,H) expression. (**I-Y**) Embryos treated at different developmental stages (as indicated) with DMSO (I,M,Q,U), with the inhibitor of RA signalling pathway DEAB (J-L,R-T) or with RA (N-P,V-Y), and analyzed for *Irx1* (I-P) or *Irx3* (Q-Y) expression. Both genes negatively or positively responded to DEAB (J,R) or RA (N,V), respectively, only when the drugs were added at stage 12.5.

Irx genes are required to maintain the kidney anlage before pronephros morphogenesis

Irx1 and *Irx3* show dynamic expression patterns during pronephros development. We find that Irx genes are initially expressed in the pronephric territory at neurula stage. This occurs after the initial specification of this territory by *Ors*, *Lim1* and *Pax8* genes at gastrula stage, but before morphological or molecular signs of kidney morphogenesis at late neurula-early tailbud sages. *Irx1* is initially activated in the dorsal pronephric territory. By contrast, *Irx3* is initially expressed in most of the kidney anlage, but it becomes confined to the ventral pronephros territory. This dorsal-ventral subdivision of the prospective kidney may reflect the initial subdivision of the pronephric territory by Notch signalling into a dorsal region that will generate glomus and proximal tubule, and a ventral domain that will give rise to distal tubule and duct (McLaughlin et al., 2000; Taelman et al., 2006). However, we have not detected an alteration of *Irx1* or *Irx3* expression by manipulating Notch signalling (not shown), which suggests that Notch does not regulate Irx genes. Interestingly, the expression of Irx genes slightly precedes the onset of expression of *Dl1* and *Notch1*, which is compatible with Irx genes participating in the regulation of Notch signalling in the pronephros. Indeed, Irx genes play a pivotal role in the regulation of Notch signalling during *Xenopus* neural crest formation (Glavic et al., 2004) and during *Drosophila* eye development (Domínguez and de Celis, 1998). The relationship between Irx genes and Notch signalling is currently under investigation.

Consistent with their initial activation in the pronephric field during neurulation, Irx function is dispensable for the initial activation of the early kidney determinants *Ors1*, *Ors2*, *Pax8* and *Lim1* that occurs at late gastrula (Carroll and Vize, 1999; Heller and Brandli, 1999; Tena et al., 2007). However, depletion of *Irx1* or *Irx3* impairs the expression of all kidney genes examined at tailbud and tadpole stages. This Irx function seems to be autonomous, as downregulation of kidney genes occurs without affecting neural or other mesodermal tissues. Consistent with a requirement for Irx genes before pronephros morphogenesis, time-controlled loss or gain of Irx function during neurula, but not during tailbud stages, reduces or expands the pronephric field, respectively. Interestingly, gain of Irx function expands but does not promote ectopic expression of *Xlim1* and *Pax8*, as found when Osr genes or *Pax8* and *Xlim1* are overexpressed (Carroll and Vize, 1999; Tena et al., 2007). This suggests that Irx genes alone are unable to trigger the kidney program. Our results indicate that Irx genes are expressed and required before appearance of any sign of pronephros morphogenesis. This early Irx gene requirement for kidney development is likely to be conserved in other vertebrates as these genes are also expressed in the early kidney anlage of other vertebrates (Houweling et al., 2001; Lecaudey et al., 2005).

During neural development, Irx proteins act as repressors and downregulate Bmp signalling to allow neural plate formation (Gómez-Skarmeta et al., 2001; Itoh et al., 2002). In this work we show that Irx proteins act as activators during kidney formation. Thus, one possible mechanism of action of Irx proteins could be to

upregulate Bmp signalling, which is known to participate at many steps during vertebrate kidney formation (Cain et al., 2008). Consistent with this idea, the reduction of Bmp signalling during *Xenopus* neurula stages causes defects similar to those produced by Irx gene impairment (Bracken et al., 2008). Furthermore, we show that increasing Bmp signalling partially rescue the kidney defects observed in Irx morphant and that *Bmp7* expression is downregulated in Irx-deficient embryos. Further experiments are required to determine more precisely the interaction between the Bmp pathway and Irx genes.

Irx genes are required at later stages for proximal-distal pronephric patterning

Recently, it has been reported that, within the pronephros field, Irx genes are initially expressed at tailbud stages and that only *Irx3* is required for proximal-distal pronephric patterning (Reggiani et al., 2007). As indicated above, we detect an earlier expression (at neurula stage) of *Irx1, Irx2* and *Irx3* in that territory, and a requirement for both *Irx1* and *Irx3* for the proper development of the kidney territory before pronephric morphogenesis. As it was possible that the late *Irx3* described function might be an indirect effect of the earlier Irx requirement, we have re-examined the participation of Irx genes in proximal-distal patterning of the pronephros. Although MOs are very useful reagents to reduce gene activity, their injections into blastomeres may impair gene function from early stages and make it difficult to recognize a late requirement. To try to uncouple early and late Irx requirements, we injected low doses of Irx MOs. In these hypomorphic conditions, embryos did not show early kidney phenotypes but the late pronephric segmentation was affected. This suggests that both genes are required for this late process but does not exclude that this could be an indirect consequence of an early requirement for Irx genes. To determine Irx protein function unambiguously during proximal-distal pronephric patterning, we have used conditional loss- and gain-of-function of Irx genes. Overexpression of an inducible dominant-negative construct demonstrates that early impairment of Irx gene activity downregulates all proximal-distal markers examined. By contrast, late impairment of Irx activity prevents only intermediate tubule formation. We confirmed this dual Irx protein function by overexpressing *Irx1* or *Irx3* at neurula or tailbud stages. Early *Irx* activation expands or promotes ectopic expression of different segment markers, whereas late overexpression expands only the intermediate tubule marker *Nkcc2*. Thus, our study reveals an earlier Irx gene requirement for most of the phronephric field and also, in agreement with Reggiani et al. (Reggiani et al., 2007), a late requirement, although in contrast to this report, our data support the necessity for both *Irx1* and *Irx3* for proximal-distal patterning.

Irx genes are regulated by RA signalling

RA is required for the activation of the early kidney genes *Lim1* and *Pax8* (Cartry et al., 2006), and for the late regionalization of the pronephros (Wingert et al., 2007). We observe that the expansion of the kidney field associated with overexpression of Irx genes are similar to that found upon increasing retinoic acid activity (Cartry et al., 2006). This suggests a possible link between Irx genes and RA signalling. Indeed, we find that both *Irx1* and *Irx3* are activated by RA signalling. This is consistent with the fact that the pronephric expression of the gene encoding the RA producing enzyme *Raldh2* and the RA receptor *RARα* precedes that of the Irx genes. Thus, RA probably lies upstream of Irx genes during pronephros development. We also find that RA is necessary for Irx gene expression at late gastrula/early neurula stages, but not later. This developmental period is when RA is required for the activation of the early kidney genes *Lim1* and *Pax8* (Cartry et al., 2006), and it is well before Irx genes are initially expressed. Thus, the RA effect on Irx gene expression is likely to be indirect, probably through *Lim1* and *Pax8*. In addition, as we do not detect alteration of the *Irx* expression patterns when RA signalling is modulated just before proximal-distal patterning, RA influence on proximal-distal patterning (Wingert et al., 2007) is likely to occur as an indirect consequence of its effect on early kidney genes such as *Lim1* and *Pax8* (Cartry et al., 2006).

In a functional survey of the enhancer activity of highly conserved non-coding elements present in the *IrxB* complex (de la Calle-Mustienes et al., 2005) we identified two ultraconserved non-coding regions (UCRs) that activate expression in the pronephros, as determined in *Xenopus* transgenic experiments (de la Calle-Mustienes et al., 2005). Each UCR is located in each Irx gene cluster in relatively close proximity to *Irx1* and *Irx3*. These regulatory regions are likely to contribute to the regulation of Irx genes during kidney development by early kidney specification genes. The detailed analyses of these regions should help unravel the molecular mechanisms that control Irx gene expression during pronephros formation and to define the genetic cascade that operates during this process.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/19/3197/DC1

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